

Validated RP-HPLC Method for Concurrent Determination of Phenytoin Sodium and Clopidogrel Bisulphate in Tablet Dosage Form

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Abstract

To develop a novel, simple, economical reverse-phase high-performance liquid chromatography (RP-HPLC) method with greater precision and accuracy to quantify phenytoin sodium and clopidogrel bisulphate in tablet dosage form. Chromatographic separation was achieved on a phenomenex, Luna C₁₈ (150 × 4.6 mm), i.d. 5µm column in isocratic mode with a mobile phase consisting of acetonitrile and water with 0.1% trifluoro acetic acid (70:30) and pH 3 adjusted with ammonia at a flow rate of 0.5MI/Min. Detection was carried out at 215 nm by UV Detector. The retention time of phenytoin sodium and clopidogrel bisulphate was 3.9 Min and 5.5 Min respectively. The method was validated as per the guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Linearity was established for phenytoin sodium and clopidogrel bisulphate in the range 10–200 µg/ML, with correlation co-efficient (r^2 value) of 0.9992 and 0.9993. The recovery of phenytoin sodium and clopidogrel bisulphate was 100.67% and 101.18%. The high recovery and low relative standard deviation confirm the suitability of the proposed method for estimating the concentrations of the drug in tablet dosage forms. Validation studies demonstrated that the proposed RP-HPLC method is simple, specific, rapid, reliable and reproducible for the determination of phenytoin and clopidogrel for quality control.

Keywords: aceclofenac, clopidogrel, Phenytoin, RP-HPLC and validation

INTRODUCTION

Epilepsy is a chronic non communicable disorder of the brain that affects people of all ages. Worldwide about 50 million people have epilepsy; it is one of the most common neurological diseases globally. The phenytoin is old and prominent anti epileptic drug in market. Phenytoin sodium (PS) is a hydantoin-derivative anticonvulsant drug used primarily in the management of complex partial seizures and generalized tonic-clonic seizures. Phenytoin is also used to prevent seizures following neurosurgery. Phenytoin is believed to protect against seizures by causing voltage-dependent block of voltage gated sodium channels [1]. Additionally, phenytoin is a class 1b anti arrhythmic that can be used to treat abnormal heart rhythms when conventional options have failed or after cardiac glycoside poisoning [2]. Chemically it is 5, 5-Diphenylhydantoin sodium salt. phenytoin is an inducer of the CYP3A4 and CYP2C19 families of the P450 enzyme responsible for the liver's degradation of various drugs[3].

Clopidogrel bisulfate (CBS) is an anti-platelet drug that inhibits the ability of platelets to clump together as part of a blood clot [4]. Clopidogrel prevents blood clots by irreversibly binding to the P2Y₁₂ receptor on platelets, preventing adenosine diphosphate (ADP) from activating platelets. It belongs to a class of drugs called P2Y₁₂ inhibitors [5]. Clopidogrel chemically called as Methyl (S)- α -(2-chlorophenyl)-6, 7-dihydrothieno [3, 2-c] pyridine-5(4H)-acetate. Clopidogrel bisulfate is used to reduce the risk of heart attacks and strokes. Clopidogrel bisulfate was approved by the FDA in 1997. Clopidogrel Bisulphate is

official drug in Indian Pharmacopoeia and United State Pharmacopoeia [6, 7].

Aceclofenac (ACF) is used as an internal standard [8]. There are number of HPLC methods available for the estimation of phenytoin and clopidogrel either alone or in combination with other drugs [9-12]. From the literature survey, till date we find out there was no HPLC method reported for simultaneous estimation of phenytoin and clopidogrel in tablet dosage form. The aim of the work is to develop and validate a simple, precise, accurate RP-HPLC method for simultaneous estimation of phenytoin sodium and clopidogrel bisulphate in tablet form. The developed method applicable to quality control, drug interaction study and dose optimization purpose.

MATERIALS AND METHODS

Chemicals and reagents

The working standard phenytoin sodium, Clopidogrel bisulphate and aceclofenac were purchased from Sigma Aldrich, India. Dilantin manufactured by Pfizer Ltd, Mumbai, Aptogrel manufactured by Aurobindo pharma Ltd, Hyderabad, with label claim of phenytoin sodium 100 mg and Clopidogrel bisulphate 75 mg was purchased from local market. Acetonitrile and Methanol (HPLC grade) purchased from Himedia Laboratories Pvt Ltd, Mumbai. Trifluoro acetic acid (TFA) was purchased from Finar chemicals Ltd, Ahmadabad. The 0.45 µm nylon filters were purchased from Millipore India Pvt Ltd. Whatmann filter paper No. 41 purchased from the local market. Purified water for chromatography was obtained from Mille-Q system was used throughout an experiment.

Apparatus and chromatographic conditions

HPLC apparatus consisting of Waters 515 system with binary pump, Rheodyne manual injector with a 20 µl loop and coupled with UV detector (Waters 2489). Data was integrated by Waters Empower 2 software and used for development and evaluation of this method. Chromatographic separation on phenomenex, Luna C₁₈ column-5µm (4.6 ×150 mm). Mobile phase consist of acetonitrile and water contain 0.1% Trifluoro acetic acid in ratio of (70:30) v/v. The mobile phase filtered through 0.45 µm nylon filter and degassed in ultra sonicator bath for 30 Min prior to use. The mobile phase pumped through column with flow rate of 0.5 MI/Min in isocratic mode. Injection volume was 20 µl throughout the study. Based on the response of all the analyte the optimum wavelength 215 nm was selected. HPLC system was operated at room temperature (25 ± 2°C).

Preparation of standard solution

1mg/ML of standard stock solutions of drug substance were prepared in methanol. Prior to measurement, stock solutions of phenytoin sodium and clopidogrel bisulphate were diluted with methanol so as to prepare working standard solution of 500 µg/ml and further dilutions were made to prepare working solutions. HPLC analysis was carried out with 20 µl aliquots of various concentration of working solutions.

Assay for pharmaceutical preparation

Twenty tablets of each marketed formulation dilantin and aptogrel were accurately weighed and finely powdered separately. A quantity of the powdered tablets equivalent to 100 mg of PS and 75 mg of CBS were accurately weighed and transferred to 100 ml volumetric flask separately. 100 ml of methanol was added to each volumetric flask and extraction were performed mechanically for 20 Min and sonicated for 30 min. The dilution was made with methanol to give solution containing 100 µg/ml of PS and 75µg/ml of CBS. All measurements were repeated six times for each concentration. The nominal contents of pharmaceutical preparation were calculated using regression equation of calibration graph.

THE METHOD VALIDATION

The method was validated in accordance with the guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [13]. The parameters assessed were linearity, accuracy, Precision, Limit of detection (LOD), Limit of quantification (LOQ), Reproducibility, Robustness and System suitability.

Accuracy

Accuracy was best determined by the standard addition method. Samples of clopidogrel and phenytoin previously analyzed for active pharmaceutical ingredient (API) were added with standard drug solutions and analyzed by the proposed method. Recovery (%) and relative standard deviation (RSD) (%) were calculated for each concentration [14].

Precision

Precision was determined as both repeatability and intermediate precision, in accordance with ICH guidelines.

The repeatability of sample injection was determined as intra-day and intermediate variation. For these determinations, Repeatability studies were performed by analysis of three different concentrations of the drugs for six times on same day. The intermediate precision of the method was checked by repeating studies on three different days.

Robustness

The concept of the robustness of an analytical procedure has been defined by the ICH - It is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters. To determine the robustness of the method, experimental conditions are purposely altered and chromatographic characteristics are evaluated. The influence of small changes in chromatographic conditions such as in flow rate (±0.1 ml/min), the wavelength for detection (±2 nm) and acetonitrile content in the mobile phase (±2%), were studied.

Linearity

Linearity of the method was studied by injecting six concentration of the drug prepared in mobile phase in the range of 10 -200µg/ml of PS and CBS (10, 20, 40, 80, 120, 160, 200µg/ml) respectively in triplicate into the HPLC system keeping the injection volume constant. The peak areas were plotted against the corresponding concentration to obtain calibration graphs.

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection LOD and limit of quantification LOQ were determined based on the standard deviation of response and slope. Detection limit and quantification limit were calculated by $[(3.3 \times \sigma)/S]$ and $(10 \times \sigma)/S$ respectively. Where 'σ' is the standard deviation of the response and 'S' is slope of the calibration curve.

RESULTS AND DISCUSSION**METHOD OPTIMIZATION**

The chromatographic conditions were optimized by using different columns, different mobile phases, different flow rates, different detection wavelengths and different diluents for standard drug and marketed tablets. In this work a simple, sensitive and validated HPLC method has been developed for simultaneous estimation of Phenytoin sodium PS, clopidogrel bisulphate CBS using liquid chromatography with ultraviolet visible detection. A number of mobile phase were initially attempted to elute three components simultaneously. The main focus was to achieve sharp Gaussian shaped peak with tailing less than 1.5 and resolution greater than 1.5. In order to achieve this goal acetonitrile, and water in different proportion were used but no sharp peaks were observed. 0.1% Trifluoro acetic acid was then added to water in the mobile phase. Adjust the pH 3 with ammonia. The mobile phase has acetonitrile and water in the ratio of 70:30 v/v. It was strong enough to elute the three components with resolution greater than 5, theoretical plate greater than 3500 and tailing less than 1.5 for the two components. The best Mobile phase composition was then found to be acetonitrile and 0.1% TFA in water in the ratio of 70:30 (v/v). Under mentioned chromatographic condition sharp peaks

belonging to phenytoin sodium, clopidogrel bisulphate and aceclofenac were obtained at retention time of 3.9, 5.5 and 7.6 Minutes respectively. [Figures 1 and 2]

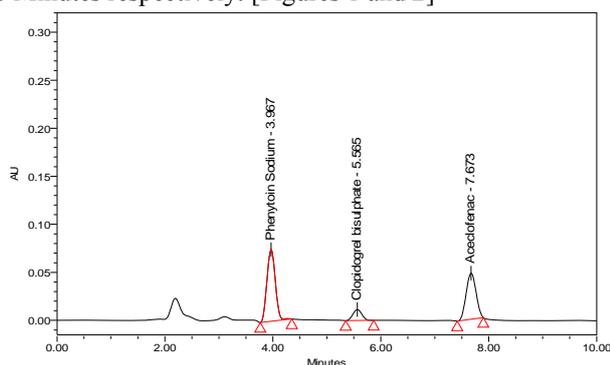


Figure 1: Chromatogram for Standard Phenytoin Sodium and Clopidogrel Bisulphate

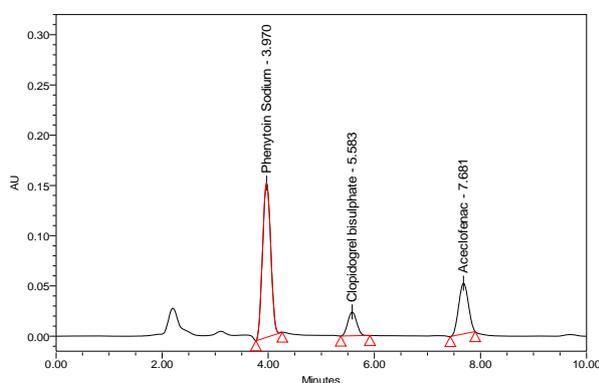


Figure 2: Chromatogram for Sample Phenytoin Sodium and Clopidogrel Bisulphate

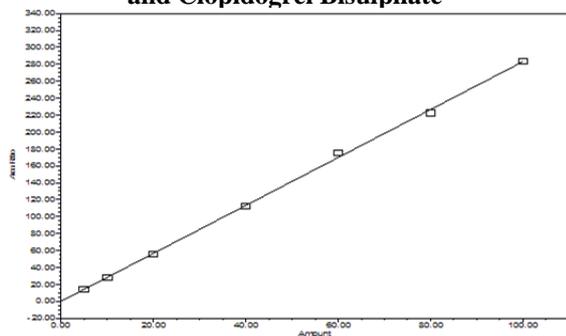


Figure 3: Calibration Curve of Phenytoin Sodium

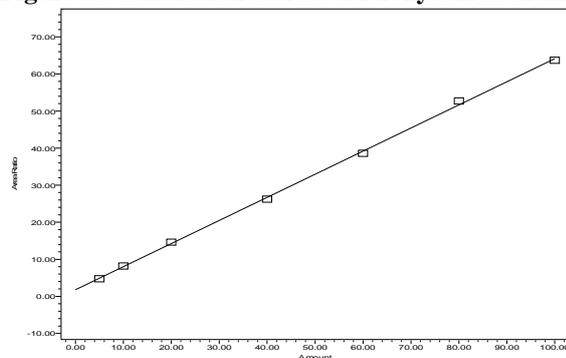


Figure 4: Calibration Curve of Clopidogrel Bisulphate

METHOD VALIDATION

Linearity

Linearity was studied by preparing standard solution at different concentration levels. The linearity range for PS and CBS were found to be 10-200 $\mu\text{g}/\text{ml}$ respectively. The regression equation for PS and CBS were found to be $Y = 2.83e+000 X - 1.09e-001$ and $Y = 6.23e-001 X + 1.78e+000$ with correlation co-efficient (r^2) 0.9992 and 0.9993 respectively for all the compounds [Figures 3 and 4]; the coefficients of determination prove that the method was linear in specific range.

Precision

The result of the repeatability and intermediate precision of the experiments are shown [Table.1]. The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were $< 2\%$, respectively as recommended by ICH guidelines.

Accuracy

The accuracy of the method was performed by the standard addition technique [14]. Three levels of solution were made which corresponds to 80, 100, and 120 % of the nominal analytical concentration. Each level was made in triplicate. The recovery and relative standard deviation for each of the analytes are given [Table 2]. From the recovery studies it is evidence that the method is highly accurate and can give excellent results.

Limit of quantification and Limit of detection

Limit of quantification (LOQ) and Limit of detection (LOD) can be determined based on visual evaluation, signal-to noise approach and standard deviation of the response and slope. The LOD of PS is $0.147821\mu\text{g}/\text{ml}$ and CBS is $0.671484\mu\text{g}/\text{ml}$, LOQ of PS is $0.447943\mu\text{g}/\text{ml}$ and CBS is $2.034\mu\text{g}/\text{ml}$ was determined.

Specificity

The peak purity of PS and CBS were assessed by comparing the retention time of standard PS and CBS. Good correlation was obtained between the retention time of standard and sample of PS and CBS. Thus the method was specific for PS and CBS.

Robustness

Robustness of the method was performed by small deliberate changes in the optimized chromatographic condition such as flow rate ($\pm 0.1\text{ml}/\text{min}$), the wavelength for detection ($\pm 2\text{nm}$) and acetonitrile content in the mobile phase ($\pm 2\%$). The result shown, that have negligible effect on retention time, recoveries and peak area of two drugs indicating the developed method is robust.

Stability

The samples of Phenytoin sodium and Clopidogrel bisulphate stored in -20°C over a period of 30 days. It did not report any difference from initial concentration. The stability of the solution is studied by performing an experiment by compare the changes in retention time, separation and asymmetry of peak in chromatogram of freshly prepared solution.

Analysis of commercial formulation

The amount of PS and CBS per tablet was calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated six times with tablet formulation. The result of analysis of tablet formulation is reported [Table 3].

Table 1: Precision of the Proposed HPLC Method

Drug	Concentration (µg / ml)	N	Within Day		Between Day	
			Mean	RSD% ^a	Mean	RSD% ^a
Phenytoin	50.2	6	50.60	1.17	50.68	1.29
	100.12	6	101.45	0.88	100.64	1.21
	150.21	6	151.44	1.01	150.48	1.00
Clopidogrel	50.42	6	50.53	1.76	50.78	1.19
	100.32	6	100.55	1.13	100.81	1.00
	150.18	6	150.99	1.02	150.78	0.90

^a RSD: Relative standard deviation**Table 2: Accuracy of the Proposed HPLC Method**

Drug	Level	Concentration (µg / ml)	Amount Recovered (µg / ml)	%Recovered	%RSD ^a
Phenytoin	80	80	81.15	101.1	1
	100	100	100.67	100.67	0.96
	120	120	121.46	101.22	1.14
Clopidogrel	80	80	80.80	101.00	1.51
	100	100	101.18	101.18	1.25
	120	120	120.88	100.73	1.12

^a RSD: Relative standard deviation**Table 3: Assay of Commercial Tablets**

Drug	Lable Claim mg	Drug Content (% ± SD)	% RSD ^a
Phenytoin	100mg	100.67± 0.96	0.96
Clopidogrel	75 mg	101.18±1.26	0.125

^a RSD: Relative standard deviation, SD: Standard deviation

CONCLUSION

New RP-HPLC method for the assay of phenytoin and clopidogrel in pharmaceutical dosage form is reported. The method is simple, reliable, linear, accurate, sensitive and reproducible as well as cost effective for quantitative analysis for phenytoin and clopidogrel in tablet formulation. The method was validated as per guidelines and all parameter are tested. The method is free from interference from other ingredients and additives used in formulation. The method showed linearity in the range 10-200µg/ml with regression coefficient of phenytoin sodium and clopidogrel bisulphate is 0.9992 and 0.9993. The recovery is nearly 100%. The RSD was < 0.1%, it is added advantage. In previous studies single drug and combination with other drug were assayed. Here we analysed two different formulations in simultaneous method. The method is suitable for use in routine quality control of phenytoin and clopidogrel in pharmaceutical dosage form.

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